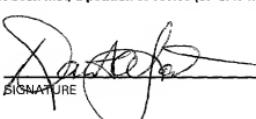


FORM-PTO-1390 (Rev. 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER
<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>		1379-1-013	
INTERNATIONAL APPLICATION NO. PCT/ES99/00017		INTERNATIONAL FILING DATE January 23, 1999	U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) <b>097600848</b>
TITLE OF INVENTION <b>PROMOTER AND REGULATOR SEQUENCES ha ds10 G1: A GENE LEA OF SUNFLOWERS EXPRESSED EXCLUSIVELY IN SEEDS FROM THE MATURATION PHASE</b>			
APPLICANT(S) FOR DO/EO/US <b>Maria Pilar PRIETO-DAPENA; María Concepción ALMOGUERA ANTOLINEZ; Juan Bautista JORDANO FRAGA</b>			
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:			
<p>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371.</p> <p>3. <input checked="" type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1).</p> <p>4. <input type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</p>			
<p>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2))</p> <p>a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</p>			
<p>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)).</p>			
<p>7. <input checked="" type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))</p> <p>a. <input checked="" type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</p> <p>b. <input checked="" type="checkbox"/> have been transmitted by the International Bureau.</p> <p>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</p> <p>d. <input type="checkbox"/> have not been made and will not be made.</p>			
<p>8. <input checked="" type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).</p>			
<p>9. <input checked="" type="checkbox"/> An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).</p>			
<p>10. <input checked="" type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).</p>			
<p><b>Items 11. to 16. below concern other document(s) or information included:</b></p>			
<p>11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98.</p>			
<p>12. <input type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.</p>			
<p>13. <input checked="" type="checkbox"/> A <b>FIRST</b> preliminary amendment.</p> <p><input type="checkbox"/> A <b>SECOND</b> or <b>SUBSEQUENT</b> preliminary amendment.</p>			
<p>14. <input checked="" type="checkbox"/> A clean copy of the Specification which includes the amendments made during the International Phase.</p>			
<p>15. <input type="checkbox"/> A change of power of attorney and/or address letter.</p>			
<p>16. <input checked="" type="checkbox"/> Other items or information:</p> <p>International Search Report; International Preliminary Examination Report; Written Opinion; Five (5) Sheets of Drawings</p>			
<p><b>EXPRESS MAIL "MAILING CERTIFICATE NO.": EL629424118US DATE OF DEPOSIT: JULY 24, 2000</b></p>			

U.S. APPLICATION NO. (If known, see 37 CFR 1.401) <b>09/600848</b>	INTERNATIONAL APPLICATION NO. PCT/ES99/00017	ATTORNEYS DOCKET NUMBER 1379-1-013																				
17. <input checked="" type="checkbox"/> The following fees are submitted:		CALCULATIONS PTC USE ONLY																				
<b>Basic National Fee (37 CFR 1.492(a)(1)-(5)):</b> Search Report has been prepared by the EPO or JPO ..... \$840.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... \$870.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(e)(2)) ..... \$760.00 Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$970.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)(4) ..... \$ 96.00																						
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		\$ 970.00																				
Surcharge of <b>\$130.00</b> for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)). <input type="checkbox"/> 20 <input type="checkbox"/> 30 <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">Claims</th> <th style="text-align: left; padding: 2px;">Number Filed</th> <th style="text-align: left; padding: 2px;">Number Extra</th> <th style="text-align: left; padding: 2px;">Rate</th> </tr> </thead> <tbody> <tr> <td style="padding: 2px;">Total Claims</td> <td style="padding: 2px;">24-20 =</td> <td style="padding: 2px;">4</td> <td style="padding: 2px;">X \$ 18.00</td> </tr> <tr> <td style="padding: 2px;">Independent Claims</td> <td style="padding: 2px;">2-3 =</td> <td style="padding: 2px;">0</td> <td style="padding: 2px;">X \$ 78.00</td> </tr> <tr> <td colspan="2" style="padding: 2px;">Multiple dependent claim(s) (if applicable)</td> <td colspan="2" style="padding: 2px;">+\$260.00</td> </tr> <tr> <td colspan="2" style="padding: 2px; text-align: right;"><b>TOTAL OF ABOVE CALCULATIONS =</b></td> <td colspan="2" style="padding: 2px; text-align: right;">\$ 1,042.00</td> </tr> </tbody> </table>			Claims	Number Filed	Number Extra	Rate	Total Claims	24-20 =	4	X \$ 18.00	Independent Claims	2-3 =	0	X \$ 78.00	Multiple dependent claim(s) (if applicable)		+\$260.00		<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$ 1,042.00	
Claims	Number Filed	Number Extra	Rate																			
Total Claims	24-20 =	4	X \$ 18.00																			
Independent Claims	2-3 =	0	X \$ 78.00																			
Multiple dependent claim(s) (if applicable)		+\$260.00																				
<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$ 1,042.00																				
Reduction for 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed. <input type="checkbox"/> (Note 37 CFR 1.9, 1.27, 1.28). <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">SUBTOTAL =</th> <th style="text-align: right; padding: 2px;">\$ 1,042.00</th> </tr> </thead> </table>			SUBTOTAL =	\$ 1,042.00																		
SUBTOTAL =	\$ 1,042.00																					
Processing fee of <b>\$130.00</b> for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)). <input type="checkbox"/> 20 <input type="checkbox"/> 30 <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">TOTAL NATIONAL FEE =</th> <th style="text-align: right; padding: 2px;">\$ 1,042.00</th> </tr> </thead> </table>			TOTAL NATIONAL FEE =	\$ 1,042.00																		
TOTAL NATIONAL FEE =	\$ 1,042.00																					
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property + <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">TOTAL FEES ENCLOSED =</th> <th style="text-align: right; padding: 2px;">\$ 1,042.00</th> </tr> </thead> </table>			TOTAL FEES ENCLOSED =	\$ 1,042.00																		
TOTAL FEES ENCLOSED =	\$ 1,042.00																					
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">Amount to be: refunded</th> <th style="text-align: right; padding: 2px;">\$</th> </tr> </thead> </table>			Amount to be: refunded	\$																		
Amount to be: refunded	\$																					
<table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="text-align: left; padding: 2px;">charged</th> <th style="text-align: right; padding: 2px;">\$</th> </tr> </thead> </table>			charged	\$																		
charged	\$																					
a. <input checked="" type="checkbox"/> A check in the amount of \$ <u>1,042.00</u> to cover the above fees is enclosed. b. <input type="checkbox"/> Please charge my Deposit Account No. <u>11-1153</u> in the amount of \$ <u>      </u> to cover the above fees. A duplicate copy of this sheet is enclosed. c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account <u>11-1153</u> . A duplicate copy of this sheet is enclosed.																						
<b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b>																						
SEND ALL CORRESPONDENCE TO: <b>DAVID A. JACKSON KLAUBER &amp; JACKSON 411 HACKENSACK AVENUE 4TH FLOOR HACKENSACK, NEW JERSEY 07601</b>																						
 SIGNATURE NAME <u>David A. Jackson</u> REGISTRATION NUMBER 26,742																						

EXPRESS MAIL "MAILING CERTIFICATE NO.": EL629424118US DATE OF DEPOSIT: JULY 24, 2000

Applicant or Patentee: María Pilar PRIETO-DAPENA, María Concepción ALMOGUERA ANTOLINEZ and  
Juán Bautista JORDANO FRAGA

Application or Patent No.: 09/600,848

Filed or Issued: July 24, 2000

For: **PROMOTER AND REGULATOR SEQUENCES Ha ds10 G1: A GENE LEA OF SUNFLOWER EXPRESSED EXCLUSIVELY IN SEEDS FROM THE MATURATION PHASE**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS  
(37 C.F.R. §§ 1.9(f) AND 1.27) - NONPROFIT ORGANIZATION**

I hereby declare that I am

the owner of the small business concern identified below:  
 an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN      1.- CONSEJO SUPERIOR DE INVESTIGACIONES CIENTÍFICAS

ADDRESS OF CONCERN      1.- Serrano, 117  
E-28006 MADRID  
Spain

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 37 C.F.R. § 1.9(d), and reproduced in 37 C.F.R. § 1.9(d), for purposes of paying reduced fees under Sections 41(a) and 41(b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average, over the previous fiscal year of the concern, of the persons employed on a full-time, part-time, or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled **PROMOTER AND REGULATOR SEQUENCES Ha ds10 G1: A GENE LEA OF SUNFLOWER EXPRESSED EXCLUSIVELY IN SEEDS FROM THE MATURATION PHASE**

by inventor(s) María Pilar PRIETO-DAPENA, María Concepción ALMOGUERA ANTOLINEZ and Juán Bautista JORDANO FRAGA

described in

the specification filed herewith  
 Application No. 09/600,848 filed July 24, 2000  
 Patent No. \_\_\_\_\_ issued

If the rights held by the above-identified small business concern are not exclusive, each individual, concern, or organization having rights to the invention is listed below, \* and no rights to the invention are held by any person, other than the inventor, who would not qualify as an independent inventor under 37 C.F.R. § 1.9(c), or by any concern that would not qualify as either a small business concern under 37 C.F.R. § 1.9(d) or a nonprofit organization under 37 C.F.R. § 1.9(e).

\* NOTE: Separate verified statements are required from each named person, concern, or organization having rights to the invention averring to their status as small entities. (37 C.F.R. § 1.27.)

NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_  
[ ] individual [ ] small business concern [ ] nonprofit organization

NAME \_\_\_\_\_

ADDRESS \_\_\_\_\_  
[ ] individual [ ] small business concern [ ] nonprofit organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earlier of the issue fee and any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 C.F.R. § 1.28(b).)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code; and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

□ NAME OF PERSON SIGNING OJEDA GARCÍA, Pedro

□ TITLE OF PERSON OTHER THAN OWNER Service chief

□ ADDRESS OF PERSON SIGNING Secano, 113

SIGNATURE Pedro Ojeda García DATE 27-07-00



in transgenic plants are demonstrated through examples such as studies related to the accumulation and location of RNAm Ha ds10 in the homologous system. Said studies show both the high expression levels reached during embryogenesis from the early maturation phases and the absolute specificity of the seed, together with a homogeneous location in embryos which is finally restricted essentially to the soft tissue in the palisade of the cotyledons, a tissue specialized in the accumulation of reserve substances in the sunflower.--.

IN THE CLAIMS:

In Claim 1, line 3, after "consisting of" delete "identical";  
line 4, before "SEQ", delete "of".

In Claim 6, line 1, delete "any of the claims 1 to 6" and insert --Claim 1--.

In Claim 14, line 1, delete "any";  
line 2, delete "of claims 1 to 13" and insert --Claim 1--.

In Claim 16, line 1, delete "any of claims" and insert --Claim--;  
line 2, delete "to 15".

In Claim 17, line 1, delete "any of claims 1 to 15"; and insert --Claim 1--.

In Claim 18, line 1, delete "any of claims 9 to 11" and insert --Claim 9--.

In Claim 19, line 1, delete "any of claims 11 to 13" and insert --Claim 11--.

In Claim 20 line 1, delete "any";  
line 2, delete "of claims 1 to 15" and insert --Claim 1--.

In Claim 22, line 1, delete "any of claims 20 to 21" and insert --Claim 20--.

In Claim 24, line 1, delete "any of claims 23 and 24" and insert --Claim 23--.

REMARKS

The above amendments are submitted herewith to add the Abstract to the Specification and to reduce the multiple dependencies and to conform the claims more closely to U.S. practice.

The amendments made herein are with respect to Claims 1-24, which claims were amended during the pendency of the International Application and accepted by the International Authority. To assure that the most current and accurate copy of the instant application is placed before the Examiner for substantive consideration, Applicants submit herewith a full copy of the International Application which include all the amendments made during the International Phase. The changes made during International processing are believed to be appropriate and are not believed to raise the issue of new matter, and entry and favorable consideration and substantive examination thereof is accordingly requested.

Respectfully submitted,



DAVID A. JACKSON  
Attorney for Applicant(s)  
Registration No. 26,742

KLAUBER & JACKSON  
411 Hackensack Avenue  
Hackensack, NJ 07601  
(201) 487-5800

## TITLE

PROMOTER AND REGULATOR SEQUENCES *Ha ds10 G1*: A GENE LEA OF SUNFLOWER EXPRESSED EXCLUSIVELY IN SEEDS FROM THE MATURATION PHASE.

5

## TECHNICAL SECTOR

Agriculture. The subject of this invention is related to obtaining of regulatory ("promoter") DNA sequences and the construction of new chimeric genes, using these sequences, capable of being specifically expressed in

10 transgenic plant seeds. *Ha ds10 G1* gene has the peculiarity of only being expressed in sunflower seeds from the maturation until the desiccation phase, without responding to hormones such as abscisic acid (ABA) or water stress in 15 vegetative tissues. Furthermore, gene *Ha ds10 G1* is expressed homogeneously in immature embryos and preferentially in the palisade parenchyma of mature embryo cotyledons. These expression patterns, as well as the high activity levels of the gene, suggest that its regulatory sequences are particularly appropriate for the genetic manipulation of storage substances in seeds

## PRIOR ART

20 Up to now in order to confer specific expression in transgenic plant seeds, promoters have been isolated, characterised and used, especially belonging to plant genes which code for storage proteins or other products solely expressed in seeds during different phases of development [see the following bibliographical references and patents, as well as other documents cited in them: Thomas TL, in 25 *Plant Cell*, vol 5, pp 1401-1410, 1993; Gatehouse JA and Shirsat AH in *Control of Plant Gene Expression*, pp 357-375, CRC press, 1993; and the USA patents numbers 5530192, 5530194 and 5420034]. For example, this has allowed the obtaining of new transgenic plants with modified fatty acid and storage protein content [see: Voelker TA, Worrell AC, Anderson L, Bleibaum J, Fan C, Hawkins 30 DJ, Radke SE and Davies HM, in *Science*, vol 257, pp.72-74, 1992, and Saalbach I, Pickardt T, Machemehl F, Saalbach G, Schieder O, and Muntz K, in *Molecular and General Genetics* 242: 226-236, 1994]. Other promoters with different tissue specificity in seed and varied temporal expression patterns could be useful for the development of the enormous potential of this technique 35 Recently in our group, and in other laboratories, we described the expression in

seeds of genes that code for low molecular weight heat shock proteins (sHSPs: *small heat-shock proteins*). One of these genes, *Ha hsp17.7 G4*, shows in tobacco transgenic plants, expression patterns appropriate for its possible use in the genetically engineered modification of seeds: this gene is expressed from 5 early seed maturation phases, and is cotyledon tissue specific [Coca MA, Almoguera C, Thomas TL and Jordano J, in: *Plant Molecular Biology* 31: 863-876, 1996]. However, gene *Ha hsp17.7 G4*, like other sHSP plant genes expressed in seeds, is also expressed in response to heat (heat shock) in plant 10 vegetative tissues after seed germination. The latter makes its use in genetic engineering impossible when regulatory DNA sequences that guarantee the absence of expression of chimeric genes outside of the seed are required: for example, when the expression elsewhere of these genes may affect viability, growth or the health of the transgenic plants. To solve these problems we modified the *Ha hsp17.7 G4* gene regulatory sequences such that the chimeric 15 genes that contain these sequences maintain their expression in seeds and lose their heat induction; a procedure which can be used for the modification and similar use of regulatory sequences of other sHSP genes expressed in seed [Almoguera, Prieto-Dapena and Jordano, patent request #9602746 (Spanish Patent Office)]. Alternatively, we have also proposed a similar use for the 20 promoter and regulatory sequences of the sunflower gene *Ha hsp17.6 G1*, that is only expressed in seeds. This gene does not respond to heat or other types of stress (cold, dehydration, ABA hormone treatment) in vegetative tissues [Carranco, Almoguera and Jordano, patent request #9701215 (Spanish Patent Office)].

25 In this application we propose alternative analogous uses for promoter and regulatory sequences of sunflower LEA *Ha ds10 G1* gene. Gene *Ha ds10 G1* has been found in a genomic clone corresponding to a previously described cDNA (*Ha ds10*, access number X506999) whose expression patterns were not totally known [Almoguera and Jordano, *Plant Mol. Biol.* 19:781-792, 1992]. The 30 promoter and regulatory sequences of this gene (*Ha ds10 G1*) have been cloned and are described, characterised and used for the first time in the examples in this application. The *Ha ds10 G1* gene belong to the Class I LEA (Late Embryogenesis Abundant) gene family (D-19 or LEA-I type) These genes code for highly conserved proteins in various plant species, and their expression is 35 usually restricted to seeds and early germination phases [see for example the

following reviews: Dure III, L., *Structural motifs in Lea proteins*, in *Plant Responses to Plant Dehydration During Environmental Stress.*, Close TJ and Bray EA Eds., *Current Topics in Plant Physiology* 10: 91-103, 1993; and Delseny M, Gaubier P, Hull G, Saez-Vasquez J, Gallois P, Raynal M, Cooke R, Grellet F,

5      *Nuclear Genes expressed during seed desiccation: relationship with responses to stress*, in *Stress-induced Gene Expression in Plants* (Basra, A. S., ed.), pp. 25-59, Harwood Academic Publishers, Reading, 1994]. LEA gene promoters have not been considered as good candidates for their use in seed storage substance modification projects, as in general their activity is expressed in later seed

10     maturation phases, such as embryo desiccation [see the considerations of Kridls JC, Knauf VC, Thompson GA in *Control of Plant Gene Expression*, pp. 481-498, CRC press, 1993]. However, LEA genes that are activated in maturation phases prior to desiccation are known, such as the cotton genes denominated LEA-A [Hughes DW and Galau GA, *The Plant Cell* 3:605-618, 1991]. Examples of

15     activation prior to desiccation are also known with the class I LEA genes, such as in the case of *At Em1*, *emb564* and *emb1* genes [in arabidopsis, maize and carrot, respectively: Gaubier P, Raynal M, Hull G, Huestis GM, Grellet F, Arenas C, Pages M, and Delseny M, *Mol. Gen. Genet.*, 238: 409-418, 1993; Williams B, and Tsang A, *Plant Mol. Biol.*, 16: 919-923, 1991; Wurtele ES, Wang H,

20     Durgerian S, Nikolau BJ, and Ulrich TH. *Plant Physiol.* 102:303-312, 1993]. These examples seem to indicate the possible use of regulatory sequences from genes in this family for the modification of seeds. However, its specific use would be limited both by the expression levels obtained in each case and in each development phase; as well as the different tissue specificities. Thus, even

25     though in *Arabidopsis* the *At Em1* gene is activated early, its expression is basically restricted to cotyledon provascular tissue and cortical tissue external to the embryonic axis [Gaubier, P., Raynal, M., Hull, G., Huestis, GM., Grellet, F., Arenas, C., Pages, M., and Delseny, M., *Mol. Gen. Genet.*, 238: 409-418, 1993]. In the case of the carrot gene, *emb1*, its mRNA are preferentially localised in the

30     embryonic meristems, especially in the *procambium* [Wurtele ES, Wang H, Durgerian S, Nikolau BJ, and Ulrich TH. *Plant Physiol.* 102:303-312, 1993]. No gene sequence of the *emb564* gene has been published and the exact localisation of its mRNA is unknown [Williams B and Tsang A, *Plant Mol. Biol.*, 16: 919-923, 1991]

35     The expression of sunflower gene *Ha ds10 G1*, as well as its promoter and

regulatory sequences present unique characteristics among the other members of the LEA-I family, as described below, which means that these sequences may be potentially used for the modification of seeds by genetic engineering.

## 5 DESCRIPTION OF THE INVENTION

In this invention we isolate and characterise in transgenic tobacco plants, the promoter and regulatory sequences of a sunflower LEA-I gene, *Ha ds10 G1*.

These sequences (Example 1) present highly appropriate characteristics for their use in the modification of seeds (e.g. storage substances). The advantages of

10 their possible use in transgenic plants are demonstrated through other examples:

A.- Studies of *HA ds10* mRNA accumulation and localisation in the homologous system (Example 2). These studies demonstrate both the high expression levels

reached during embryogenesis from early maturation phases, as well as the absolute seed specific localization, accompanied of a homogenous distribution in  
15 embryos which terminates essentially restricted to the cotyledon palisade parenchyma, a tissue specialised in the accumulation of sunflower storage substances. B.- In example 3, we also illustrate the possible use of such

20 sequences via the construction and analysis of various chimeric genes in transgenic plants, using the promoter and combinations of various *Ha ds10 G1* regulatory sequences (5'-flanking, coding, intron and 3'-flanking), with the reporter gene of bacterial  $\beta$ -glucuronidase (GUS). These examples demonstrate in a heterologous model (tobacco) the usefulness of the different chimeric genes tested: high expression level and specificity to seeds from early maturation phases, as well as the functional contribution of the various sequences tested.

25 Via the examples attached we demonstrate that the seed specificity is basically conferred by the promoter and the 5'-flanking sequences of *Ha ds10G1* (including untranscribed and transcribed sequences: such as the 5'-UTR and part of the coding sequence). Additionally, the 3'-flanking sequences increase expression levels in seeds and the intron specifically reduces it in non-embryonic tissues.

30 Given the conservation of the regulation of embryonic gene expression in plant seeds, including LEA-I genes [Thomas TL, in I 5:1401-1410, 1993]; these sequences could be used both in the homologous system (sunflower) as in other heterologous systems of great economic importance (for example oilseed rape, soybean, maize, etc).

35 The practical embodiment of this invention, represented by the attached

examples and figures, uses conventional Molecular Biology, Microbiology, recombinant DNA and transgenic plant production techniques that are common practice in laboratories specialised in these fields. These techniques have been explained in sufficient detail in the scientific literature [for example see: Sambrook

5 J, Fritsch EF, and Maniatis T, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor laboratory Press, 2nd Edition, 1989; Glover DM, *DNA Cloning*, IRL Press , 1985, Lindsey K., *Plant Tissue Culture Manual*, Kluwer Academic Publishers, 1993; and Gelvin SB, Schilperoort RA, Verma DPS, *Plant Molecular Biology Manual*, Kluwer Academic Publishers , 1992]. For more specific details,  
10 the pertinent bibliographical references are cited in the corresponding section in this application.

EXAMPLE 1: Cloning, determination of restriction map, nucleotide sequence and analysis of the *Ha ds10 G1* promoter.

To obtain the *Ha ds10 G1* clone the sunflower genomic DNA gene library described by Coca *et al.* [Plant Mol. Biol. 31: 863-876, 1996] was screened, with the probe corresponding to total *Ha ds10* cDNA [Almoguera and Jordano, Plant Mol. Biol. 19: 781-792, 1992]; using standard hybridisation conditions and molecular cloning procedures described in detail in the first of the references (Coca *et al.*, 1996). We thus isolated a phage (IGEM11) with a sunflower genomic DNA insert of approximately 16.5 Kb whose partial map is shown in Figure 1. We determined, using restriction analysis, that the two fragments adjacent to the Sac I site (4.2 and 9.3 Kb) contain the sequences that hybridise with the cDNA. A detailed restriction map of the first of these fragments was determined and part (4 Kb) of the second (Figure 1). Different genomic DNA subfragments, corresponding to the mapped region, were cloned in pBluescript SK+ vector, resulting in plasmids whose names and inserts are listed in Figure 1. The 3617 bp nucleotide sequence between the Sac I and Sma I sites (Figure 1, lower section) was determined from these plasmids on both DNA strands using the Sanger (dideoxy) method. These data are presented in SEQ No. 1. We confirmed by comparing the sequences, that part of the genomic sequence determined corresponds to *Ha ds10* cDNA [Almoguera and Jordano, Plant Mol. Biol. 19: 781-792, 1992, GenBank access number X59699]. The amino acid sequence of the protein coded by the *Ha ds10 G1* gene is indicated below the corresponding nucleotide sequences. In the genomic DNA, the coding region is interrupted by an abnormally long intron (1024 bp), even though it is situated in a

conserved position in other class I LEA genes [see data reviewed by Simpson GC, Leader DJ, Brown JWS and Franklin T, in *Characteristics of Plant pre-mRNA Introns and Transposable Elements, Plant Mol. Biol. LabFax*, pp 183-252; Croy RRD Ed., Bios Scientific Publishers Ltd. 1993]. The only difference between the 5 gene sequences coding for mRNA and those of cDNA, was a two nucleotide inversion (GC instead of CG) within the second exon (in positions +1176 and +1177 from the initiation codon) which induces an amino acid change (S instead of T) in the protein sequence. The difference is due to an error (due to a compression) in the initial reading of the cDNA sequence reactions. The *Ha ds10* 10 *G1* sequences we have determined also include 1576 bp of the gene promoter and 5'-flanking region, and 553 bp of 3'-flanking genomic regions not present in the original cDNA.

Three possible transcription initiation sites were determined in the *Ha ds10* *G1* promoter by the primer extension technique. Two of these sites have been 15 confirmed with other techniques (sites 1 and 2, indicated by arrows in SEQ No. 1). For this the procedure described by Domon *et al.* was used [Domon C, Evrard JL, Pillay DTN and Steinmetz A. *Mol. Gen. Genet.* 229:238-244, 1991], total sunflower embryo RNA was hybridised with the synthetic primer: 5'-CTCCTGTTCCGGAATTTGCGTGT-3', whose sequence corresponds to that of 20 the non coding strand of *Ha ds10 G1*, between positions +25 and +48, from the initiation codon. The hybridisations with the primer were carried out at 62°C. The hybrids were extended with AMV reverse transcriptase, for 90 min at 42°C. The extension products were analysed on 6% PAGE sequencing gels, along with sequence reactions produced using the same primer. Initiation sites 1 and 2 (at 25 positions -33 and -25, see SEQ No. 1) are functional, and are detected independently using the ribonuclease A protection technique (RNase A, see Figure 3A). A third initiation site (site 3, in position -119 in SEQ No. 1) could not be clearly confirmed with this technique. These initiation sites functionally define the 3' end of the *Ha ds10 G1* gene promoter.

30 The analysis of the proximal sequences of the *Ha ds10 G1* gene promoter demonstrated that the two initiation sites detected (sites 1 and 2) are found at an appropriate distance from a possible TATA sequence (at position -86). The possible more distal site (site 3, -119) does not have clear TATA sequences in its proximity. Apart from these promoter elements, two possible RY "boxes" (RY1 35 and RY2 at positions -129 and -65 of SEQ No. 1) were observed, analogous to

those that participate in the regulation of the expression of numerous plant genes in seeds [Dickinson DC, Evan RP, and Nielsen RC, in *Nucleic Acids Research* 16: 371, 1988 ].

We modified the RY1 box sited at position -129, verifying by transient expression experiments in sunflower embryos, its functional requirement for the trans-activation of the *Ha ds10 G1* promoter by ABI3 type transcription factors [Graudat J., Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM in *The Plant Cell*: 1251-1261, 1992]. In order to do this we prepared modifications of the ds10::GUS fusions constructed for transgenic plant studies (see Example 6.3 and Figure 5). The chimeric genes contained in these two fusions (ds10F1 and ds10F2) are purified as DNA fragments which were subcloned by ligation into pBluescript SK+ (Promega) vector, thus changing the binary vector sequences for smaller ones, more useful for transient expression experiments. We thus obtained the plasmid pSKds10F1 using the Sal I - Eco RI fragment (with the 15 chimeric gene obtained from ds10F1). In the case of ds10F2, the Sph I - Eco RI fragment (from position -125 in *Ha ds10 G1*, to the 3' end of *nos*) was ligated to the complementary fragment (which contains the promoter and 5'-flanking sequences of *Ha ds10 G1*), purified after digestion of pSKds10F1 with Sph I and Eco RI, resulting in the pSKds10F2 plasmid. Finally, from the pSKds10F1 and 20 pSKds10F2 plasmids (maps not shown) mutagenised versions were obtained by digestion of their DNA with Sph I, blunting the resulting ends by treatment with T4 DNA polymerase, followed by re-ligation of the DNA. We thus obtained plasmids pSKds10F1 $\Delta$ RY and pSKds10F2 $\Delta$ RY (maps not shown). These plasmids only differ by a 5 nucleotide deletion between positions -126 and -122 of the *Ha ds10 G1* promoter. These changes destroyed the RY1 box present in the ds10F1 and ds10F2 chimeric genes (see Figures 1, 2 and 5), this was verified by the Sanger (dideoxy) method sequencing reactions, using the primer 25 5'CTCCTGTTCCCGAATTTGCGTGT3' (non coding strand of *Ha ds10G1* between positions +25 and +48).

30 The trans-activation experiments in transient expression were carried out by bombarding sunflower embryos with projectiles coated with DNA mixtures from different plasmids. These mixtures contain a reference plasmid, pDO432 [Ow DW, Wood KV, deLuca M, de Wet JR, Helinski D and Howell SH. *Science* 234: 856-859, 1996], with the firefly (*Photinus pyralis*) luciferase (LUC) gene 35 regulated by the CaMV 35S promoter, the fusion of ds10::GUS tested in each

case (with intact or modified RY1 sequences), and an effector plasmid, pABI3, which expresses the ABI3 factor under control of the CaMV 35S promoter. pABI3 was obtained by substituting the Pv ALF cDNA from the pALF plasmid [Bobb AJ, Eiben HG, an Bustos MM in *The Plant Journal* 8: 331-343, 1995], with ABI3 cDNA. The ABI3 cDNA was cloned as an Xba I fragment (blunted with Klenow enzyme) - Eco RI (partial), fragment purified from the pcabi3-4F plasmid [Giraudat J., Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM in *The Plant Cell* 4: 1251-1261, 1992]. pABI3 plasmid is added to, or omitted from, the mixture to test the effect of the ABI3 factor on GUS expression in the fusion tested. The 10 experiments were essentially carried out as described by Bobb *et al.*, [Bobb AJ, Eiben HG, and Bustos MM in *The Plant Journal* 8: 331-343, 1995], with the following modifications. Sunflower embryos (17-20 dpa) were prepared as follows. Sunflower seeds were sterilised by washing in 70% ethanol for 1 min, and in 2% sodium hypochlorite with a drop of Triton X-100 for 40 min, finally 15 rinsed several times with distilled water, and then peeled under sterile conditions. The embryos are cut longitudinally (separating the two cotyledons) and placed, with the cut surface down on MS solid medium plates, containing 2% sucrose and 0.5 M sorbitol. They are then pre-cultured for 2-4 h in the dark at room temperature (25°C). All the plasmids were purified using the *Quantum midiprep* 20 *kit* (Biorad). Normally for each bombardment were used: 0.2 µg of reference plasmid, 0.1 µg ds10::GUS plasmid and 1 µg of effector plasmid (or the same amount of pJIT82 plasmid in the negative controls). For the preparation of the gold particles, as well as the DNA precipitation onto them, we followed the method described by Chern *et al.* [Chern MS, Bobb AJ and Bustos M. *The Plant Cell* 8: 305-321, 1996]. The particle bombardment was carried out using the *Biostatic PDS-1000 He* system (Biorad). The bombardment conditions were the following: 1550 psi rupture membrane, 1.6 µm diameter gold particles, distance from rupture membrane to macrocarrier 8 mm, distance from macrocarrier to grid 6 mm, and distance to the tissue to be bombarded 6 cm. The bombarded 25 cotyledons were incubated for 24 h at 28 °C in the dark, after which the GUS activity (relative to LUC activity) was tested as described by Bobb *et al.* [Bobb AJ, Eiben HG, and Bustos MM in *The Plant Journal* 8: 331-343, 1995].

The addition of pABI3 effector plasmid had a clear effect on the relative expression of GUS/LUC in bombarding with the pSKds10F2 fusion (average 35 increase in relative activity ≈ 46.2X). On the other hand, if the trans-activation

was carried out with the same plasmid with a mutation in the RY box (pSKds10F2 $\Delta$ RY1), a significant reduction in the average increase in relative activity due to the ABI3 effect ( $\approx 26.3X$ ) was observed. This result, shown in figure 2, confirms the functional requirement of the RY1 sequence (position -129 in SEQ No. 1). Therefore, this RY box participates in the transcriptional activation in seeds of the *Ha ds10 G1* promoter for ABI3 type factors [Giraudat J., Hauge BM, Valon C, Smalle J, Parcy F, Goodman HM in *The Plant Cell* 4: 1251-1261, 1992]. Other promoter sequences (e.g. RY2 in -65) could also contribute to the transactivation effect observed, as the mutation tested does not completely 10 destroy the activator effect of ABI3.

EXAMPLE 2: Accumulation and specific localisation of *Ha ds10* mRNA in sunflower embryos:

The messenger RNA accumulation patterns of the *Ha ds10G1* gene were 15 determined by the Ribonuclease A (RNase) protection technique , described in detail by Almoguera *et al.* [Almoguera C, Coca MA, Jordano J. *Plant Physiol.* 107: 765-773, 1995]. To do this, total RNA samples prepared from seed embryos at different stages of development under normal growth conditions were used [Almoguera and Jordano, *Plant Mol. Biol.* 19: 781-792, 1992; Coca *et al.*, *Plant Mol. Biol.* 25: 479-492, 1994]; of seedlings 3-day after imbibition (dpi); and of 20 different adult plant organs before flowering. The seedling and plant RNA were prepared from plant material obtained both under controlled growth conditions [Almoguera and Jordano, *Plant Mol. Biol.* 19: 781-792, 1992; Coca MA, Almoguera C, and Jordano J. *Plant Mol. Biol.* 25: 479-492, 1994; Coca MA, 25 Almoguera C, Thomas TL, and Jordano J. *Plant Mol. Biol.* 31: 863-876, 1996], and after stress treatments: water deficit [Almoguera C, Coca MA, and Jordano J. *Plant J.* 4: 947-958, 1993; Coca MA, Almoguera C, Thomas TL, and Jordano J. *Plant Mol. Biol.* 31:863-876, 1996]; or after addition of hormones such as abscisic acid [Almoguera C and Jordano J. *Plant Mol. Biol.* 19: 781-792, 1992; Coca MA, 30 Almoguera C, Thomas TL, and Jordano J. *Plant Mol. Biol.* 31: 863-876, 1996]. The conditions used in each treatment are described in detail in the references cited for each case. The riboprobe used to detect the *Ha ds10 G1* mRNA is 396 nucleotide long, of which 63 are sequences of the pBluescript SK+ vector and the rest the sequence of the non coding strand of *Ha ds10 G1* between positions 35 +212 and -121 (Sph I). This hybrid probe with the 5' end of *Ha ds10 G1*

messenger RNAs, exceeding the more distal transcription initiation site (site 3, SEQ No. 1), allows the detection of messenger RNA (mRNA) produced from the three initiation sites and the experimental verification of the initiation positions. This riboprobe was prepared by *in vitro* transcription, using RNA polymerase T3 5 and as a template ds10G1S3Δ4.4 plasmid DNA (Figure 1) which contains the *Ha ds10G1* sequences between -1576 (Sal I) and +212 cloned in the pBluescript SK+ vector.

The results in Figure 3 show that the *Ha ds10 G1* messenger RNAs are only detected in seeds. Higher accumulation levels are observed around 18-20 10 dpa, gene expression is detected from 10 dpa and it disappears after germination (Figure 3). Treatments with ABA, or water deficit did not induce the accumulation 15 of *Ha ds10 G1* messenger RNAs (data shown for ABA in seedlings, Figure 3). As a positive control in the RNA samples tested for the different treatments, we carried out hybridisations (data not shown) with another previously described 651 nucleotide riboprobe of *Ha hsp17.7 G4* gene [Coca *et al.*, *Plant Mol. Biol.* 31: 863-876, 1996]; as this gene is expressed in response to the different treatments tested. These analysis showed that the *Ha ds10 G1* mRNAs were only accumulated in seeds, under normal growth conditions and from early stages of 20 maturation, confirming the initiation from at least sites 1 and 2 (indicated in SEQ No. 1). The band marked by the number 3 (Figure 3) does not coincide well with the expected size for initiation site 3 (SEQ No. 1). This band could be due to the protection of messenger RNA sequences of a highly homologous gene, or even *Ha ds10 G1* itself, containing intron sequences (unprocessed mRNA).

The distribution of *Ha ds10 G1* mRNAs in sunflower embryos was 25 investigated by *in situ* hybridisation localisation experiments. In order to do this, embryos were embedded in paraffin, fixed, sectioned and hybridised with specific probes; essentially as described by Molinier [in the thesis: *Diplome d'Etudes Approfondies de Biologie Cellulaire et Moléculaire, Université Louis Pasteur, Strasbourg, 1995*]. The fixing time was increased from 16 h at 4°C to 5 days, the 30 increase depending on the age of the embryos. The dehydration of the fixed embryos was carried out by successive incubations (2 times each for 30-90 min.) in 10%, 20%, 30%, 40%, 60%, 70%, 95% and 100% ethanol; followed by immersion in 100% toluene (1-3h, 2 times). The fixed embryos were first embedded in toluene:paraffin (1:1), at 65°C for 6-15 h, followed by 5 consecutive 35 inclusions in paraffin, at 60°C for 5-15 h. The pre-hybridisations and

hybridisations with the probes were carried out at 45°C. The specific *Ha ds10 G1* riboprobe, corresponding to the mRNA 3' end was prepared as follows: The *ds10G1S1* plasmid (Figure 1) was used as a template to prepare two *in vitro* transcription probes [Almoguera C, Coca MA and Jordano J. *Plant Physiol.* 107: 765-773, 1995] marked with DIG-UTP. The *ds10-3'(-)* is obtained by digesting plasmid DNA with *Pvu* II and carrying out the transcription with RNA polymerase T3. This probe corresponds to the non-coding strand of *Ha ds10 G1* between positions +1202 (*Pvu* II in the second exon) and +1592 (3' end). The second probe [*ds10-3' (+)*, used as a control], was prepared digesting *Ha ds10 G1S1* DNA with *Bam* HI (in the *polylinker*); and carrying out the transcription with RNA polymerase T7. Probe *ds10-3'(+)* contains the coding chain of *Ha ds10 G1*, between position +870 and +1592. The specificity of the hybridisation was determined by Southern blot experiments similar to those described by Almoguera and Jordano [Plant Mol. Biol. 19: 781-792, 1992]. While the hybridisation with a total cDNA probe detects bands corresponding to some 4-5 different genes in the sunflower genome [Almoguera C, and Jordano J. Plant Mol. Biol. 19: 781-792, 1992]; using probe *ds10-3'(-)* we can detect a single gene (with a slight cross hybridisation with another one, data not shown).

The results obtained in the RNA localisation experiments are shown in

Figure 4. Probe *ds10-3'(-)* is complementary and has opposite polarity to *Ha ds10 G1* mRNA, which allows its detection. The results obtained agree with the protection data shown in Figure 3, and demonstrate its accumulation in embryos from 12-15 dpa (Figure 4A) to 21-28 dpa (Figures 4C, F and H). This accumulation takes place to high levels, which can be deduced from the short time required for its histochemical detection (2-4 hours). In immature embryos (Figure 4A) the distribution of *Ha ds10 G1* mRNA is homogeneous and comparable (Figure 4B) to that of 18S rRNA, which is detected using another riboprobe corresponding to fragment G (*Eco* RI) of the radish 18S gene [described by Delcasso-Tremousaygue D, Grellet F, Panabieres F, Ananiev E D, and Delseny, M. in Eur. J. Biochem. 172: 767-776, 1988]. In more mature embryos (21 dpa, Figure 4C) the *Ha ds10 G1* mRNA are also localised fairly homogeneously, with a more intense accumulation detected in the vascular bundles (*procambium*), something which is not observed with the 18S rRNA probe nor in this or other development stages (Figures 4D, B and G). Finally, at 28 dpa the *Ha ds10 G1* mRNA are preferentially localised in the palisade .

parenchyma, a tissue specialised in the accumulation of storage substances, located in the internal face of cotyledons (Figures 4F and H). The localisations with probe ds10-3' (+), with the same polarity as the *Ha ds10 G1* mRNA, did not give any hybridisation signal, which was a control for the previously described experiments (compare Figures 4C and E). These experiments demonstrated that the *Ha ds10 G1* mRNA expression patterns in sunflower are very special. The expression observed in seeds, with high levels of accumulation from early embryonic maturation stages (10-12dpa), are combined with spatial distributions which change from homogeneity to a greater abundance in storage substance deposit tissues (palisade parenchyma). The distribution and accumulation pattern of *Ha ds10 G1* mRNA is different from that presented by other plant genes belonging to the same family [Wurtele ES, Wang HQ, Durgerian S, Nikolau BJ and Ulrich TH. *Plant Physiol.* 102: 303-312, 1993; Gaubier, P., Raynal, M., Hull, G., Huestis, GM., Grellet, F., Arenas, C., Pages, M., and Delseny, M. *Mol. Gen. Genet.*, 238: 409-418, 1993]. These results indicate the potential usefulness of chimeric genes that incorporate *Ha ds10 G1* regulatory sequences for the modification of seeds by genetic engineering.

**EXAMPLE 3: Construction of ds10G1::GUS chimeric genes and their analysis in tobacco transgenic plants.**

As an example of the possible uses of the promoter and the regulatory sequences of *Ha ds10 G1* gene in the construction of chimeric genes with specific expression in transgenic plant seeds, we describe below the construction and analysis of 4 ds10G1::GUS translational fusions in tobacco transgenic plants (Figure 5). These fusions contain the promoter and different combinations of flanking and intragenic sequences of *Ha ds10 G1* gene for its functional analysis. These 4 fusions provide high levels of expression of the reporter gene (GUS) in seeds from early maturation stages (Figure 6), confirming our observations in the homologous system (Example 2, Figures 1-4).

The first of these constructions, ds10F1 (Figure 5) was obtained from the ds10G1S3 plasmid (Figure 1), which contains the genomic sequences of *Ha ds10 G1* between Sal I (-1576) and Eco RI (+1086), subcloned into the corresponding restriction sites of the pBluescript SK+ vector (Promega). The *Ha ds10 G1* sequences between Eco RI (+1086) and position +98 (in the first exon) were deleted by treating with Exonuclease III the ds10G1S3 DNA (previously digested

with Hind III and Pst I), resulting in ds10G1S3Δ10.5 plasmid (Figure 1). This plasmid was digested with Bam HI (*polylinker* restriction target situated immediately adjacent to position +98 of *Ha ds10 G1*), then filling in the digested DNA ends using the Klenow fragment of DNA polymerase I. The DNA was then 5 digested with Sal I, and the 1679 bp fragment containing the *Ha ds10 G1* sequences between Sal I (-1576) and the filled end of Bam HI was purified. This fragment was cloned between the Sal I and Sma I sites of the pBI 101.2 binary vector, resulting in ds10F1, a translational fusion which contains 1576 nucleotides of 5'-flanking *Ha ds10 G1* sequences (from ATG) and the first 98 nucleotides of 10 the coding gene, in phase with the GUS gene (Figure 5). The ds10F2 fusion was derived from ds10F1 by the insertion of a genomic DNA fragment of *Ha ds10G1* comprised between positions (Figure 1) +1205 (*Pvu* II) and *Eco* RI ( $\approx$ +4670) . This fragment contains part of the second exon and  $\approx$ 3370 nucleotides of 3'- 15 flanking sequences (from the termination codon in position +1301); and replaces the nos-3' sequences in the ds10F1 fusion. The *Pvu* II- *Eco* RI insert was purified 20 from ds10G1S2 plasmid DNA. For the insertion of this fragment, the ds10F1 DNA was digested with *Sac* I and the DNA ends were blunted by treating with T4 DNA polymerase I. Then, the DNA thus treated was digested with *Eco* RI, and the fragment including the *Ha ds10G1* sequences was purified. This fragment was 25 ligated to the previously described *Pvu* II- *Eco* RI insert (with the *Ha ds10 G1* 3'-flanking sequences), resulting in the ds10F2 fusion (Figure 4). The ds10F2Δ fusion (Figure 4) was obtained from ds10F2, by the deletion of the *Ha ds10G1* 3'-flanking sequences between *Xba* I ( $\approx$ +2830) and *Eco* RI ( $\approx$ +4670). To do this, ds10F2 DNA was digested with both enzymes, religating after blunting the 30 resulting DNA ends with the Klenow fragment of DNA polymerase I. Finally, the fourth fusion (ds10F3, Figure 5) was obtained from a *Ha ds10 G1* genomic DNA fragment between Sal I (-1576) and *Pvu* II (+1204), purified from ds10G1S6 plasmid (Figure 1) after digestion with both restriction enzymes. This fragment was ligated with vector pBI101.3 vector, previously digested with Sal I and Sma I. 35 The ds10F3 fusion thus contains the promoter and the same 5'-flanking sequences of *Ha ds10 G1* present in ds10F1 fusion, as well as the first exon (From +1 to +145), the total intron (from +146 to +1169) and part of the second exon of *Ha ds10 G1* (from +1170 to +1204), fused in phase with the pBI 101.3 GUS gene. In all cases the nucleotide sequence corresponding to the fusion zone, between the GUS and the *Ha ds10 G1* sequences, was tested by

sequencing reactions with the Sanger (dideoxy) method, using GUS sequences as the primer: 5'-ACCGCGCTTCCCACCAACGCTG-3'.

The T-DNA in ds10F1, ds10F2, ds10F2 $\Delta$  and ds10F3 fusions (Figure 5) was mobilised from *A. tumefaciens* (LBA 4404), obtaining different tobacco

5 transgenic plants with independent integrations of each chimeric gene. These plants were obtained and characterised by standard techniques as described in detail by Coca MA, Almoguera C, Thomas TL and Jordano J, [in *Plant Molecular Biology*, 31: 863-876, 1996]. The expression of GUS gene was analysed both in developing seeds and under normal growth conditions (without exogenous

10 stress); as in seedling tissues, in the latter case the expression changes induced by ABA and dehydration treatments were studied. The seed analysis were carried

out with the original transgenic plants (T0), while those of the seedlings used descendants of these plants (T1), segregating for the chimeric genes. Quantitative studies by fluorometric analysis of GUS expression levels and their

15 temporal patterns, as well as qualitative studies which analysed histochemically the spatial patterns of expression (tissue specificity) were carried out. These studies were carried out as described in detail by Coca MA, Almoguera C,

Thomas TL and Jordano J, [in *Plant Molecular Biology*, 31: 863-876, 1996]. In total, the following number (in parenthesis) of tobacco transgenic plants, T0

20 "functional", containing the chimeric genes ds10F1 (14), ds10F2 (7), ds10F2 $\Delta$  (8) and F3 (23) were obtained and analysed. These plants showed high levels of

GUS gene expression in seeds (as a result of the activity of the *Ha* ds10 G1 gene promoter and regulatory sequences), as illustrated in Figure 6 (panels A-C). The integration of the different chimeric genes in the transgenic plants' DNA was

25 characterised by *Southern* analysis using probes for the coding GUS gene region; PCR amplifications of the sequences close to the ds10::GUS splice, using the 5'-ACCGCGCTTCCCACCAACGCTG-3' (GUS) and 5'-

GAGTGAAACAgAATtcCATCACAAACAGGG-3' (ds10Eco RI) primers; or by the Kanamycin resistance segregation test (conferred by the *nptII* gene), performed

30 as described in [Jordano J, Almoguera C, and Thomas TL, *The Plant Cell* 1: 855-866, 1989]. These analysis determined that the T0 plant selected for the seed expression studies contained 1 to 5 integrations independent of the corresponding chimeric gene. Figure 6 (joined to this application) illustrates the

more relevant results obtained in the study of the expression of the chimeric genes analysed in transgenic plants. These results are described in detail below.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35

GUS expression during seed maturation under controlled growth conditions (without exogenous stress), was analysed by fluorometric (Figure 6A) and histochemical (summary in Figures 6B-E) assays. The fluorometric assays were carried out in seeds at defined maturation stages, 12, 16, 20, 24 and 28 days post-anthesis (dpa). For each T0 plant and maturation stage, two different floral capsule extracts were prepared, and the GUS activity was assayed with Methylumbelliferylglucuronide (MUG) in duplicate (in total four activity determinations per development stage and per individual transgenic plant). The statistical significance of the differences observed with the different GUS fusions was determined, after log normalisation of the data obtained, by variance analysis [ANOVA, see: Nap JP, Keizer P, and Jansen R, in *Plant Molecular Biology Reporter* 11: 156-164, 1993]. The histochemical assays were carried out with material dissected from seeds, at defined development stages, from the following number of transgenic plants: d10F1, 5, ds10F2, 6, ds10F2Δ, 6 and dsF3, 19. The 10 endosperm and the embryos dissected from individual seeds were stained with X-gluc, for 150 min at 25°C, approximately 150 seeds from each transgenic plant 15 were analysed in this manner.

All chimeric genes produced high levels of GUS expression in seeds, reaching average maximum values of  $1.65 \times 10^6$  pmol MU/ mg x min (Figure 6A: 20 at 24 dpa). The histochemical assays confirmed these high activity values, since both the embryos (Figures 6B and C) and the endosperm (Figure 6C) were strongly stained from 12 dpa (Figure 6B) and with only 150 min of reaction. In both cases fairly homogeneous spatial distributions of the GUS activity were observed (Figure 6B-C). Furthermore, these expression patterns do not differ 25 qualitatively between the different chimeric gene transgenic plants (data not shown).

The fluorimetric assays revealed interesting quantitative differences between the different ds10::GUS fusions. These differences depend on the *Ha ds10 G1* sequences present in the fusions. In some cases the statistical 30 significance of these differences could be demonstrated (with a confidence level of 95%), which experimentally demonstrates the contribution of the different sequences tested (promoter and 5'-flanking sequences, coding sequences, 3'-flanking and intron) to the embryonic expression patterns observed. The presence of *Ha ds10 G1* 3'-flanking sequences in the fusions increases the GUS 35 expression levels in seeds between 20 and 28 dpa (compare fusions ds10F2 and

ds10F2 $\Delta$ , with ds10F1 in Figures 5 and 6A). This difference is statistically significant (for example at 28 dpa:  $F = 5.397$ ,  $P: 0.0213$ ), and is caused by the *Ha ds10 G1* sequences present in the ds10F2 $\Delta$  fusion (see Figure 5); since no significant differences were found between the GUS activity of ds10F2 and 5 ds10F2 $\Delta$  (for example, also at 28 dpa,  $F=0.274$ ,  $P=0.6015$ ; see Figure 6A). In the case of ds10F2 $\Delta$ , the stimulating effect of the 3'-flanking sequences also occurs and is highly significant, in earlier embryonic maturation development stages (Figure 6A, 16 dpa;  $F=16.607$ ,  $P=0.001$ ). On the other hand, in these stages (between 12 and 16 dpa) ds10F1 and ds10F2 GUS activities do not differ 10 significantly (e.g. at 16 dpa:  $F=2.762$ ,  $P=0.0983$ ; see Figure 6A). Overall these results show that ds10F2 $\Delta$  is the constructed and tested fusion that works the best in tobacco seeds from 16dpa; and that this is due to the effect of *Ha ds10 G1* 3'-flanking sequences included in it. We do not know if this effect is caused by transcriptional activation or mRNA stabilisation mechanisms, or by a combination 15 of both. In any case the effect is clear and the potential usefulness to design new chimeric genes with more efficient expression in seeds, from relatively early embryonic maturation stages (see also the section "Other Examples").

On the other hand, the comparison of the GUS activities in plants with the ds10F1 and ds10F3 fusions allowed us to investigate the possible effects of the 20 presence of the intron (and/or *Ha ds10 G1* coding sequences in which these fusions differ, Figure 5) on the expression of both fusions. In transgenic tobacco seeds these comparisons demonstrate that the presence of the intron (plus the first total exon and part of the second exon) does not have positive effects on GUS expression, which must be therefore essentially conferred by the *Ha ds10 G1* promoter and the sequences present in ds10F1 (Figure 6A). Thus for 25 example, the activities of ds10F1 and ds10F3 are not statistically different between 12 and 28 dpa, except at 20 dpa ( $F=4.73$ ,  $P=0.031$ ) and then the presence of additional sequences in ds10F3 significantly reduced the GUS activity observed. Therefore, even though it is highly probable that the intron is 30 correctly processed in the seeds of heterologous systems such as tobacco (we do not have any formal proof), its possible regulatory role in embryonic development is unclear. However other observations do not exclude that the additional *Ha ds10 G1* sequences in ds10F3 (including the intron) may have regulatory roles in other tissues (see below the effect of these sequences on 35 residual expression of ds10::GUS fusions in pollen and seedlings).

Embryonic specificity (to seeds) of GUS expression conferred by the *Ha ds10 G1* sequences in tobacco transgenic plants was verified through investigations in other tissues; both in the absence of stress as well as after dehydration and ABA treatments. In the case of T0 plants, the only tissue where GUS activity was detected by fluorimetric and histochemical assays, was mature pollen. In other tissues the activities detected barely exceeded background levels (non-transformed tobacco plants). For example, in T0 plant leaves of about two months of age: 0-50 pmol MU/ mg x min. The activities detected in pollen are marginal (almost three orders of magnitude less) when compared with those of seeds from the same transgenic plants. Furthermore, this expression could be an artefact and depend on the use of GUS gene as an indicator in the fusions [according to Uknes S, Dincher S, Friedrich L, Negrotto D, Williams S, Thompson-Taylor H, Potter S, Ward E, and Ryals J, in *the Plant Cell* 5: 159-169, 1993]. However, surprisingly we observed that the activity measured in the pollen of the 9 ds10F3 plants was (136  $\pm$ 64 pmol MU/ mg x min) significantly less than that of the 5 ds10F1 plants (6427  $\pm$ 1294 pmol MU/ mg x min; F= 72.573, P= 0.0001). The latter could indicate that, unlike what is observed in seeds during most of their embryonic development (Figure 6A), the presence of the additional *Ha ds10 G1* sequences in ds10F3 (including the intron) may reduce the expression of the chimeric genes containing them in other tissues or stages of development.

The possibility of expression of the ds10::GUS fusions being induced by hormones (ABA) or stress treatments (water deficit) in tobacco transgenic plants (T1) at different times in its vegetative cycle was also checked. In order to do this, we selected descendants of 8 different original plants, after germination in MS medium with 300  $\mu$ g/ml kanamycin, containing ds10F1, ds10F2 $\Delta$  and ds10F3; and another 6 with ds10F2. The resistant seedlings were transplanted in MS medium. Various experiments were carried out with seedlings, both at 8 and 15 days after imbibition. For the ABA treatments, the seedlings were transplanted in MS plates supplemented with 100  $\mu$ M ABA and cultivated in this medium for 4 days at 25°C in light. The control seedlings were also transplanted in MS medium without ABA. Water stress was induced by placing the seedlings for about 5-6 hours in a laminar flow hood between two filter papers. After the different treatments, the seedlings were processed either individually (for the histochemical assays with X-gluc, by 14 h incubations at 25 °C); or jointly (pool

analysis), for the GUS activity fluorimetric assays as described previously. The adult transgenic plant treatments, were carried out using individual plants propagated as vegetative clones obtained from each original plant. To do this, the seedlings selected from each transgenic plant were transplanted to vermiculite  
5 imbibed with Hoagland 0.5X medium. From each seedling three complete explants were obtained, which were placed in hydroponic culture, after recovery, in liquid Hoagland medium (0.5X). The experiments were carried out when the plants had completely recovered from the propagation process, and had roots, stem and about 10-12 leaves. Therefore, genetically identical plants from each  
10 selected transgenic seedling were used for the different treatments. The ABA treatments were carried out by adding the hormone to the medium (100  $\mu$ M) and analysing the GUS activity in the plants after 24h. Water stress was induced by removing the root from the container with the medium, also analysing the plants 24h after starting the treatment. The effect of the different treatments was  
15 assessed in three independent experiments performed with the following number of T1 plants for each fusion (the number of T0 plants from which they proceed in each case is given in parenthesis): ds10F1, 11 (6); ds10F2, 10 (5); ds10F2 $\Delta$ , 5 (3); and ds10F3, 10 (5).

The experiments carried out both in seedlings and in adult plants which  
20 confirmed the embryonic specificity of the expression conferred by the *Ha ds10 G1* sequences to the different fusions, also providing additional clues to the possible regulatory role of the *Ha ds10 G1* sequences present in ds10F3 (including the intron) previously mentioned. Thus, both in control adult plants as in treated plants minimum GUS activities (from 3 to 300 pmol MU/ mg x min)  
25 were detected in all the tissues analysed (roots, stem, leaves and apical meristem). These activity levels are only slightly above the background levels and can only be detected fluorimetrically (data not shown).

In 8 dpi seedlings the expression of all the fusions is about two order of magnitudes lower than the maximum levels reached in seeds. This expression  
30 rapidly decreases between 8 and 15 dpi (e.g. ds10F1 goes from 2864  $\pm$ 182 to 813 $\pm$ 104 pmol MU/ mg x min); and is exclusively restricted to embryonic tissue (cotyledons), without it being detected in other vegetative tissues (radicle, hypocotyl, leaves) differentiated after germination (Figures 6D and E, and data not shown for the other fusions). These results confirm in transgenic tobacco plants the embryonic specificity of the regulation by *Ha ds10 G1* sequences.  
35

Apart from the general reduction in GUS activity values mentioned previously, differences between the values of the different fusions, some statistically significant, were observed. These differences were qualitatively similar to those observed in seeds (Figure 6A). Among them, and for its possible applied interest, 5 we illustrate the reduction of expression after germination, mediated by the *Ha ds10 G1* sequences present in ds10F3 (including the intron). This effect is observed as a significant reduction of GUS activity when the ds10F1 and ds10F3 10 plant expression patterns are compared (Figures 6D and E). The statistical analysis of the quantitative ds10F1 and ds10F3 data confirmed the significance of this difference, both at 8 dpi ( $F= 4.36$ ,  $P= 0.04$ ) and at 15 dpi ( $F= 4.39$ ,  $P= 0.039$ ). Additionally, a moderate induction of GUS by ABA treatment in ds10F1 seedlings 15 was observed, which is statistically significant (from  $2864 \pm 182$  to  $5790 \pm 733$  pmol MU/ mg x min;  $F= 5.413$ ,  $P= 0.023$ ). In the case of ds10F3 there was no significant induction by the same treatment (from  $1502 \pm 195$  to  $2338 \pm 211$  pmol MU/ mg x min;  $F= 2.58$ ,  $P= 0.11$ ). The different treatments did not substantially affect the tissue specificity, or the order of magnitude of the expression observed for the different ds10::GUS fusions (data not shown).

#### OTHER EXAMPLES:

20 Other chimeric genes can be obtained, in an analogous manner to that of the one described in detail in the previous example, which contain 5'-flanking, and(or) 3'-flanking (terminators), and(or) coding sequences from *Ha ds10 G1*, combined with sequences from other genes. These examples do not involve any additional technical complications to those described in more detail in the 25 previous sections, for which reason they can be easily carried out by persons with sufficient knowledge in the sector of the invention technique. Thus for example, in ds10::GUS fusions the *Ha ds10 G1* could have included other longer 5'-flanking (Figure 1) sequences of the same gene to increase its expression level in seeds as we described in [Coca MA, Almoguera C, Thomas TL, and Jordano J, in *Plant Molecular Biology* , 31: 863-876, 1996]. Equally, the GUS sequences could be substituted by others coding for different proteins or peptides (natural or artificial), whose regulated production in plant seeds could be of industrial interest. Examples of these last possibilities, non exclusively, would be the fusion with *Ha ds10 G1* sequences of coding sequences of genes involved in fatty acid 30 biosynthesis in seeds [Voelker TA, Worrell AC, Anderson L, Bleibaum J, Fan C,

Hawkins DJ, Radke SE and Davies HM, in *Science*, 257:72-74, 1992], of storage proteins with compositions rich in specific amino acids [Saalbach I, Pickardt T, Machemehl F, Saalbach G, Schieder O, and Muntz K, in *Molecular and General Genetics* 242: 226-236, 1994], or peptides with antigenic or pharmacological activities [Vandekerckhove J, Van Damme J, Van Lijsebettens M, Boterman J, De Block M, Vandewiele M, De Clercq, Leemans J Van Montagu, M and Krebbers E, in *BioTechnology* 7: 929-932, 1989]. These fusions would be carried out and used in an analogous manner to what is described in the publications cited as an example (not exclusive) in each case. To facilitate these possibilities, 10 we have created a plasmid (ds10EC1) that contains an expression cassette including the promoter and the 5'- and 3'-flanking sequences of *Ha* ds10 G1 present in ds10F2Δ (see Figure 5). Between both sequences and by directed mutagenesis [Chen E and Przybila AE, in *BioTechniques* 17: 657-659, 1994] we have added an Eco RI restriction site, which allows the insertion of gene, or 15 corresponding peptide sequences, as mentioned previously (available in other laboratories, or that could be designed or synthesised). The ds10EC1 plasmid was constructed from ds10G1S3Δ10.5 (Figure 1). From this plasmid, we amplified the *Ha* ds10 G1 sequences between positions -1574 (Sal I) and +98 by PCR; using DNA polymerase Pfu and the primers 5'-ATTAACCCTCACTAAAG-3' 20 (T3) and 5'-GAGTGAACAgAATtcCATCACAAACAGGG-3' (ds10Eco RI). In the latter the three sequence changes (indicated in lower case letters) introduce the new Eco RI site in the position of the initiation codon. After PCR a 199 pb (*megaprimer*) DNA fragment is purified, which along with the 5'-AATACGACTCACTATAG-3' (T7) primer is used for a second PCR amplification 25 of ds10G1S3Δ10.5. The amplified DNA (795 pb) was digested with Eco RI and Sph I. The resulting DNA fragment (125 pb), with the *Ha* ds10 G1 sequences between Sph I (-126) and the new Eco RI site, was purified and ligated; replacing in ds10G1S3 the *Ha* ds10 G1 (Figure 1) sequences between positions -126 (Sph I) and 1086 (Eco RI). After this step, the PCR amplified sequence was verified by 30 sequencing (Sanger's method) using the T3 primer. Finally, an *Ha* ds10 genomic DNA fragment (Figure 1) was inserted in the plasmid obtained in the previous step, with sequences between +1086 (Eco RI) and ≈+3000 (Xba I), obtaining the ds10EC1 cassette (Figure 4), cloned in the pBluescript SK+ plasmid. The 3' end of ds10EC1 DNA differs from that of ds10F2Δ only by 119 35 additional nucleotides, corresponding to the intron and second exon sequences

of *Ha ds10 G1*. Furthermore, the *Ha ds10 G1* sequences in ds10EC1 differ from the corresponding ones in ds10F2Δ in the absence of nucleotides 1-98 of the first exon (Figure 5).

Given that the presence of additional *Ha ds10 G1* sequences in ds10F3 (including the intron, the first exon and part of the second exon) reduced the expression of this chimeric gene specifically in non embryonic tissues (Example 3, Figures 6D-E), it is conceivable that such sequences may be used to confer seed specificity to other chimeric genes with different promoters. The design of such chimeric genes does not involve additional technical difficulties other than those described in the previous sections: see for example the detailed procedures on the use of plant introns to prevent the expression of chimeric genes in *Agrobacterium* [Mankin SL, Allen GC and Thompson WF. *Plant Molecular Biology Reporter* 15: 186-196, 1997]

The chimeric genes containing the *Ha ds10G1* regulatory sequences could be transformed to other plants different from tobacco (the model system used in example 3). Among these there are plants with major economical interest such as: sunflower, soybean, oilseed rape, "canola", maize, wheat, barley, rice, cassava, bean, peanuts, etc. whose genetic transformation is possible and has been sufficiently documented in the scientific literature: see for example Lindsey K, Ed. (1993). [*Plant Tissue Culture Manual*. Kluwer Academic Publishers]; and the review by Christou [*Trends in Plant Science*. 1: 423- 431, 1996]. The results shown in example 3 demonstrate that, in tobacco, the genes constructed with the *Ha ds10 G1* regulatory sequences have a high activity from relatively early embryonic maturation stages, and also maintain the seed specificity characteristic of *Ha ds10 G1* in sunflower. These results could also be obtained with other plants, such as those mentioned previously.

#### DESCRIPTION OF THE FIGURES:

**Figure 1.** Upper section: restriction map of the *Ha ds10 G1* genomic sequences flanking its coding region. The continuous lines on the map indicate the different genomic DNA fragments subcloned in pBluescript SK+ vector (the names of the respective fragments are indicated over each fragment). The plasmids prepared by Exo III deletions are indicated over the original plasmid (ds10G1S3ΔSacI), indicating in each case the deletion end. On the lower section of the figure a detailed restriction map of the region whose nucleotide sequence was determined is shown. The extension of the different reactions used to

assemble the different sequences of both DNA strands, are indicated by horizontal arrows (above the map for the coding strand, and underneath the map for the non-coding strand). The transcription initiations sites are indicated with arrows. Scale bars are included for both maps.

5

**Figure 2.** Functional implications of the RY1 (-129) sequences in the trans-activation of the *Ha ds10 G1* promoter. Transient expression experiments carried out after bombarding the sunflower embryos with DNA coated micro-projectiles. The results of 5 independent experiments, in which the different plasmid mixtures (described in Example 1) where bombarded five times in each experiment, are presented. The average  $\beta$ -glucuronidase (GUS) activities normalised versus luciferase activity (LUC), as well as the standard error (indicated with bars), are presented. Key: F2, pSKds10F2; F2 $\Delta$ RY1, pSKds10F2 $\Delta$ RY1; AB13, samples with the effector plasmid. A significant decrease in the relative GUS/LUC activity is observed, due to a mutation in the RY1 box. The basal activities for pSKds10F1 (without including the effector plasmid) are of the order of 46 $\pm$ 8.

**Figure 3.** Accumulation patterns of *Ha ds10 G1* gene mRNA in sunflower. The autoradiograph shown corresponds to the RNase A protection tests, after hybridising a gene riboprobe with different total RNA samples. An accumulation of messenger RNA produced from *Ha ds10 G1* transcription initiation sites (as protected fragments indicated by the numbered arrows) is observed. These fragments are only detected in embryos (Emb) from 10 to 20 dpa and in mature seeds (25 dpa), but not in other samples tested, such as seedlings (Germ) or seedlings treated with ABA (Germ + ABA). The carrier tRNA corresponds to control hybridisations with yeast tRNA. The bands corresponding to the mRNAs produced from the different initiation sites are indicated with numbers and arrows. The initiation site number 3 (indicated in parenthesis) has not been experimentally confirmed by primer extension. On the left margin are included molecular size markers (pBR322/Hpa III).

**Figure 4.** Localisation of mRNA in sunflower embryos sections at 12 (A and B), 21 (C-E), and 28 dpa (F-H). The following riboprobes were used in each case: ds10 (-), A, C, F, H; ds10 (+), E, and 18S rRNA, B, D, G. Scale bar = 500  $\mu$ m (Except in F, 125  $\mu$ m). Palisade parenchyma= pp. The arrows mark the

*procambium.*

**Figure 5.** Restriction maps of ds10::GUS fusions and optimised expression ds10 EC1 cassette, constructed in Examples 3 and 4. The *Ha ds10* G1 and other genes contained in each case are shown by shading of varying intensity. The transcription initiation sites from the *Ha ds10* G1 promoter are marked with arrows.

**Figure 6.** Expression of ds10::GUS fusions in tobacco transgenic plant seeds. Panel A: Summary of quantitative data (fluorimetric determinations). The average of GUS activities in transgenic plant seeds (T0), and its evolution through the different embryonic development stages is shown. The data corresponding to each fusion are indicated by the symbols in the upper left-hand insert. The bars indicate the standard errors. Panels B-E: representative selection with results of the histochemical GUS activity localisation experiments: B.- embryos at 12 dpa (plants ds10F2Δ, T0). C.- embryos and endosperm at 16 dpa (ds10F2Δ plants, T0). D.- seedlings at 15 dpi under control conditions (ds10F1 plants, T1) E.- seedlings at 15 dpi under control conditions (ds10F3 plants, T1) In panels D and E, the arrows indicate the plant tissue without GUS activity (leaves and hypocotyl).

#### LIST OF SEQUENCES:

**SEQ No. 1:** *Ha ds10 G1* gene nucleotide sequence. The transcription initiation sites experimentally determined (site 3, which has not been confirmed by primer extension is indicated in parenthesis) are indicated by arrows. The coding zone is shown by its amino acid translation indicated by the letter (LO1 etc.) code underneath the nucleotide sequence. The termination codon is indicated by an asterisk. The sequence is numbered (on the left margin) starting from the initiation codon. The intron sequences are shown in lower case letters. The TATA box (in position -86) and RY box (-129 and -65) mentioned in the text (Example 1) are shown underlined.

## LIST OF SEQUENCES

<110> Consejo Superior de Investigaciones Cientificas

5 <120> Ha ds10G1 promoter and regulatory sequences: a LEA gene  
expressed only in seeds

<130> Ha ds10G1

10 <140>  
<141>

<160> 1

15 <170> PatentIn Ver. 2.1

<210> 1

<211> 3617

<212> DNA

20 <213> Helianthus annuus

<220>

<221> exon

<222> (1577)..(1721)

25

<220>

<221> exon

<222> (2746)..(2879)

30

<220>

<221> intron

<222> (1722)..(2745)

35

<220>

<221> misc\_feature

<222> (1448)..(1455)

<223> RY box

40

<220>

<221> misc\_feature

<222> (1512)..(1519)

<223> RY box

45

<220>

<221> polyA\_signal

<222> (3139)..(3144)

50

<220>

<221> polyA\_signal

<222> (3220)..(3225)

<400> 1

gtcgacttct tcatcttcgt ctaagtgtt agtatcgagt acaaatttattt catcttcgtt 60

gtcatacgat atgagaacga tttgttttaac attatcttt ggatattgg acgggtggact 120

ccgataaaga aacgggttca aaggctttttt ggcttcggta ggatactcgat acacacccgg 180

tgcaaccatc gtgggactttt gtggtaatgtt aaaaaactgtt ggtggatagt tgggttgggg 240

ttgggtgtttt taaaaccccg ttgtggaaa aaatgtgggg ggaatgtggg ttgttacaca 300

atagatcac ctcgttctact ttgcgagccg cggcccccctcg cattcgaaacc ggaatccgt 360

actttttttt ttttcatgac cttttttttt tctatccatg gtatgcatc gaatttggtaa 420

attgggtgaa aaatggaaatg ttaaacatgg taaaatggaa tggttataatt ataaaggat 480  
 ttaatgttt ttttaaacc ataaacggtc atatagccgt ttaaaggcaca acggtcaagt 540  
 ccccaacggt caaatcaagc caaacaaatca agtcccccgt gtggcatata tccctgtctt 600  
 tgaaccaggc cggcccaaaat cctaagaatt ttgaggctt ggctttaggc ctctaaatca 660  
 5 ataggactc taactaaaaa aattatataa tgatatttag gttaaagtta atttatcttt 720  
 atatgtcaca aaaaatataat ataaatccaa aataaaaaaaat aaaaaaataaa attaacttcg 780  
 ccaacaataa atctttttgt atgttttgc tttttttt ttaaaaaaaat gctcaattt 840  
 ttaatttgct ttggccacc aaaaatggta aaccggacctt gcttgaacc ataccacac 900  
 gacaatttag gggatcagga gttggggctg gcaaaacat gcaaaaaaac tttgcgcac 960  
 10 aaaggttcac catttcgggtt atgtttggc gatgcggcga ggaggaagaa gagagaggag 1020  
 ggtagtgtgaa ggtggggttc attccaaatc caactaaatc cattttttt cttttttttt 1080  
 tttttttttt ttaacactt actaaatgtc taaaaccatc ccaacactt tcaacaaatg 1140  
 ttaacattt ttctctgtt gacgtggcac actcttcattt gttttttttt tagtttgcac 1200  
 ctctcaacc ttaatccatc ctttacacc tttttttttt ggggtgggtt tcccaacagc 1260  
 15 aaaaaaggcc ttaaccacac cttttttttt tttttttttt tttttttttt tttttttttt 1320  
 atactgcattt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1380  
 gggacactgt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1440  
 gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1500  
 cttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1560  
 20 taaaaggatg aaaaaatatttggt cttttttttt tttttttttt tttttttttt tttttttttt 1620  
 ggagaagaaat gatcttcggc aacggcggc aacggcggc aacggcggc aacggcggc 1680  
 tcggtggccaa tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1740  
 aattttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1800  
 aaaaaaggcc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1860  
 agggggccat gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1920  
 25 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 1980  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2040  
 aacttgcaccc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2100  
 gacaaaaaaa tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2160  
 30 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2220  
 accgggtttcg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2280  
 aaaaacccgg tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2340  
 gccatgtttaa aatggatgtt atccggatgtt gttttttttt tttttttttt tttttttttt 2400  
 35 gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2460  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2520  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2580  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2640  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2700  
 40 aataatataat tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2760  
 gacaacaccc tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2820  
 gtcagacccac tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2880  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 2940  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3000  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3060  
 45 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3120  
 aaaaatgttta cggatgtacaa taaaatggcaaa tttttttttt tttttttttt tttttttttt 3180  
 gacttgcctt caataacggca ataaatgttta tttttttttt tttttttttt tttttttttt 3240  
 aacaacttattt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3300  
 agccaaataaa tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3360  
 50 aaaaatgttta aatgttgcac tttttttttt tttttttttt tttttttttt tttttttttt 3420  
 gcgatcttcg cccggacac tttttttttt tttttttttt tttttttttt tttttttttt 3480  
 aataatgggtt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3540  
 tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3600  
 gttttttttt tttttttttt tttttttttt tttttttttt tttttttttt tttttttttt 3617

**CLAIMS**

1. A nucleotide sequence constituted by the *Ha ds10 G1* gene, its promoter, *Ha ds10 G1* 5'- and 3' flanking sequences, wherein the nucleotide sequence is selected from the group consisting of identical nucleotide sequences identical to SEQ ID NO:1, first homologous nucleotide sequences being homologous by at least 70% to SEQ ID NO:1, second homologous nucleotide sequences being homologous being at least 70% homologous to complementary sequences to SEQ ID NO:1, and fragments thereof.

10 2. A nucleotide sequence according to claim 1, wherein the first homologous sequence is homologous by at least 80% to SEQ ID NO:1.

15 3. A nucleotide sequence according to claim 1, wherein the first homologous sequence is homologous by less than 95% to SEQ ID NO:1.

20 4. A nucleotide sequence, wherein the second homologous sequence is homologous by at least 80% to SEQ ID NO:1.

5. A nucleotide sequence according to claim 1, wherein the second homologous sequence is homologous by less than 95% to SEQ ID NO:1.

25 6. A nucleotide sequence according to any of the claims 1 to 6, and further including a chimeric gene.

7. A nucleotide sequence according to claim 6, suitable for expression of a chimeric gene.

30 8. A nucleotide sequence according to claim 7, wherein the chimeric gene is specifical of seeds from early maturation stages.

9. A nucleotide sequence according to claim 8, constituted by constructions *ds10F1*, *ds10F2*, *ds102Δ*, *ds10F3* and *ds10EC1* or part thereof.

35 10. A nucleotide sequence according to claim 10, including *Ha ds10 G1* gene coding and 3'-flanking sequences.

11. A nucleotide sequence according to claim 10, including ds10F2 and ds10F2 $\Delta$  in constructions.

5 12. A nucleotide sequence according to claim 8, including *Ha ds10 G1* gene coding and intron sequences.

13. A nucleotide sequence according to claim 12, contained in constructions ds10F3.

10 14. An expression cassette including a nucleotide sequence according to any of claims 1 to 13 and a chimeric gene.

15 15. A vector including an expression cassette according to claim 14.

16. Host cells including a nucleotide sequence according to any of claims 14 to 15.

20 17. Use of nucleotide sequences as defined in any of claims 1 to 15, in the specific expression of chimeric genes in seeds, seed parts, seed extract, seed embryos and seedling tissues.

25 18. Use of nucleotide sequences as defined in any of claims 9 to 11 for increasing the expression of chimeric genes specifically in transgenic plant seeds.

19. Use of nucleotide sequences as defined in any of claims 11 to 13 for increasing the expression of chimeric genes in seeds and/or reduce it in other tissues.

30 20. A transgenic plant transformed by a nucleotide sequence according to any of claims 1 to 15.

35 21. A transgenic plant according to claim 20, selected from sunflower, tobacco, soya, oilseed rape, "canola", maize, wheat, barley, rice, bean, cassava

TRANSPARENT DOCUMENT

and peanut.

22. Use of a transgenic plant according to any of claims 20 to 21 for the production of substances resulting from the expression of chimeric genes.

5

23. Use of a transgenic plant according to claim 22 wherein the substances are proteins, bioactive substances and oils.

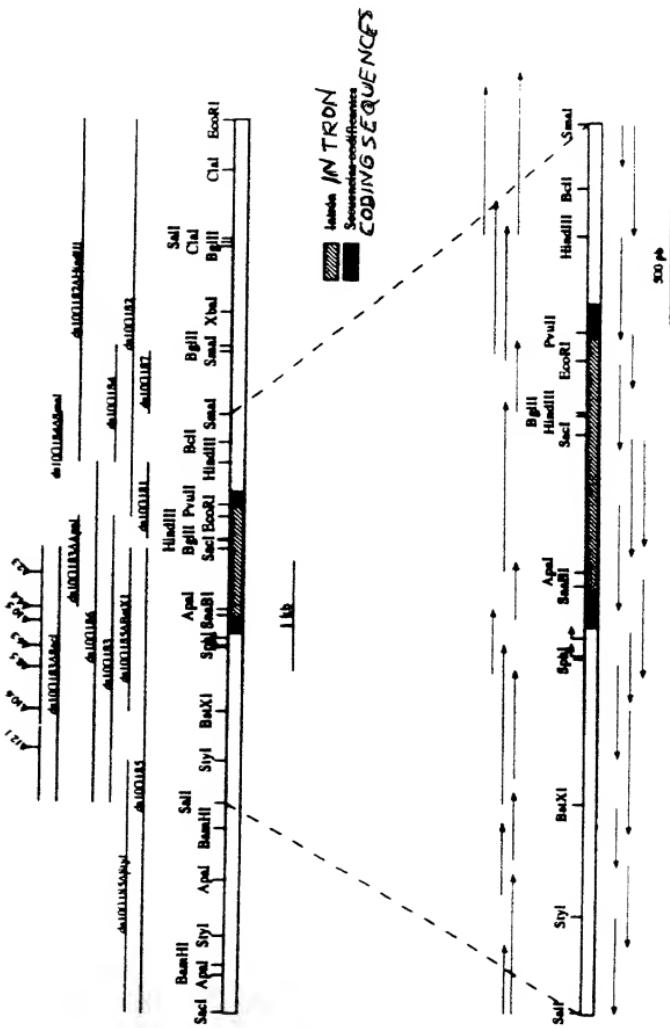
24. Substances obtained according to any of claims 23 and 24.

09/600848  
PCT Chapter II

532 Rec'd PCT/PTC 24 JUL 2000

## DRAWINGS

*The following five (5) pages comprise copies of the drawings as published but with hand-written translations into English of the Spanish text of the drawings*



**Figura 1**

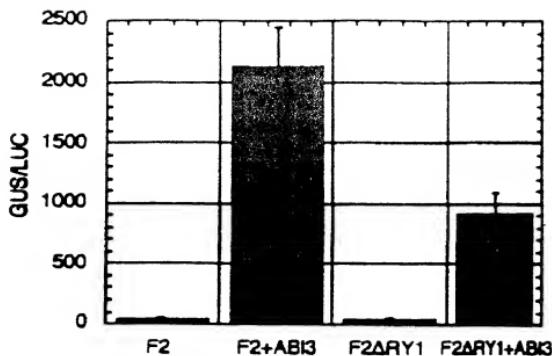


Figura 2



Figura 3

3/5

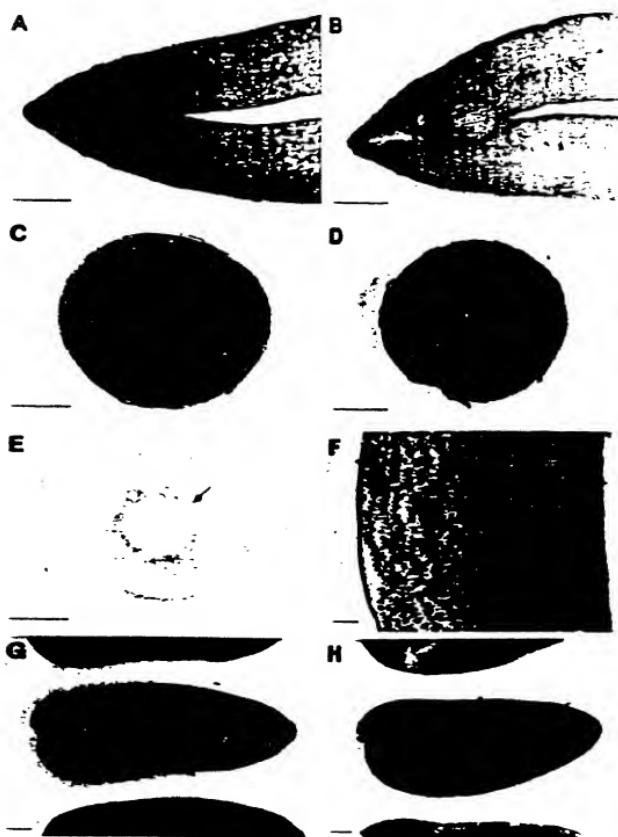
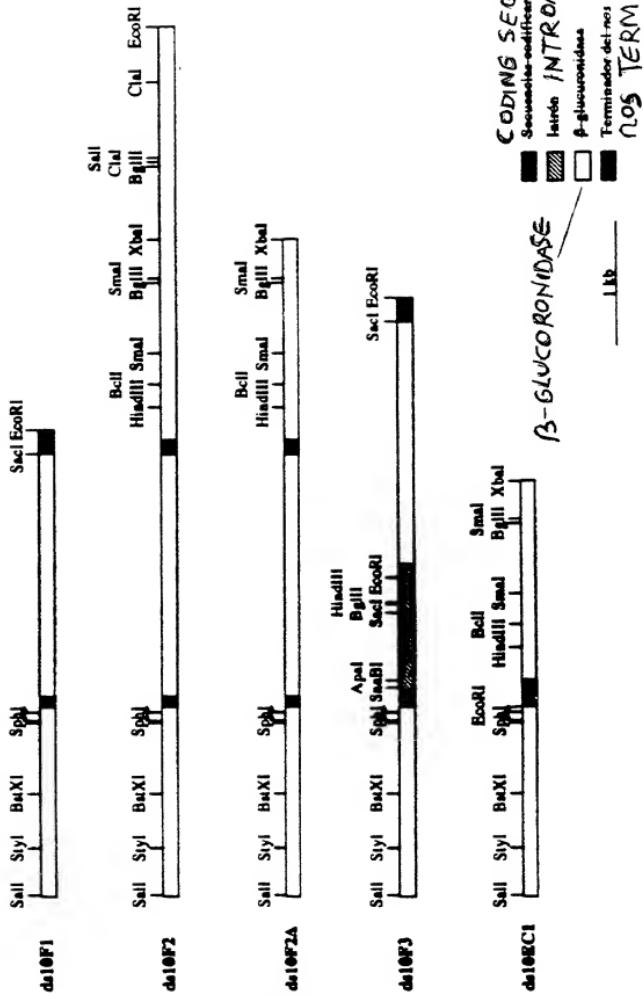


Figura 4



**Figura 5**

5/5

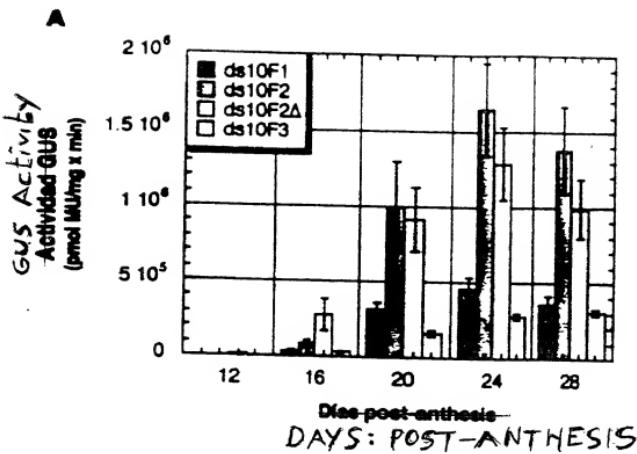
**B****C****D****E**

Figura 6

**DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION**

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below under my name.

I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

**PROMOTER AND REGULATOR SEQUENCES Ha ds10 G1: A GENE LEA OF SUNFLOWER EXPRESSED EXCLUSIVELY IN SEEDS FROM THE MATURATION PHASE**

the Specification of which

is attached hereto  
 was filed on July 24, 2000  
as Application Serial No. 09/600,848  
and was amended on (if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified Specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 (a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

**PRIOR FOREIGN FILED APPLICATION(S)**

<u>APPLICATION NUMBER</u>	<u>COUNTRY</u>	<u>(MONTH/DAY/YYYY)</u>	<u>PRIORITY CLAIMED</u>
P 9800122	SPAIN	23 January 1998	YES

I hereby claim the benefit under Title 35, United States Code §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER(S)

FILING DATE (MM/DD/YYYY)

→

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s), or §365(a) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application..

<u>U.S. Parent</u>	<u>PCT Parent</u>	<u>Parent Filing</u>	<u>Parent Patent</u>
<u>Application No.</u>	<u>Number</u>	<u>(MM/DD/YYYY)</u>	<u>Number (if applicable)</u>

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Ungria Patentes y Marcas, S.A. as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

 I hereby appoint as my attorneys or agents the following persons: Jack Matalon (Attorney, Registration No. 22,441); Stefan J. Klauber (Attorney, Registration No. 22,604); David A. Jackson (Attorney, Registration No. 26,742); Michael D. Davis (Attorney, Registration No. 39,161); Allan H. Fried (Attorney, Registration No. 31,253); Christine E. Dietzel (Agent, Registration No. 37,309); Donald J. Cox (Attorney, Registration No. 37,804); and Michael A. Yamin (Agent, Registration No. 44,414), said attorneys or agents with full power of substitution and revocation to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Please address all correspondence regarding this application to:

DAVID A. JACKSON, ESQ.  
KLAUBER & JACKSON  
411 HACKENSACK AVENUE  
HACKENSACK, NEW JERSEY 07601

Direct all telephone calls to David A. Jackson at (201) 487-5800.

 I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

*1-00*  
FULL NAME OF FIRST OR SOLE INVENTOR: PRIETO-DAPENA, Maria Pilar

COUNTRY OF CITIZENSHIP: Spain

FULL RESIDENCE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla *ESX*  
Spain

FULL POST OFFICE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla  
Spain

SIGNATURE OF INVENTOR Pilar Prieto Dapena

DATE 26-7-00

*2-00*

FULL NAME OF SECOND JOINT INVENTOR: ALMOGUERA ANTOLINEZ, María Concepción

COUNTRY OF CITIZENSHIP: Spain

FULL RESIDENCE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla *ESX*  
Spain

FULL POST OFFICE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla  
Spain

SIGNATURE OF INVENTOR Concepción Almoguera Antolines

DATE 26-7-00

*3-00*

FULL NAME OF THIRD JOINT INVENTOR: JORDANO FRAGA, Juan Bautista

COUNTRY OF CITIZENSHIP: Spain

FULL RESIDENCE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla *ESX*  
Spain

FULL POST OFFICE ADDRESS: Instituto Recursos Naturales y Agrobiología Sevilla  
Consejo Superior de Investigaciones Científicas  
Apartado 1052 Estafeta-Puerto  
E-41080 Sevilla  
Spain

SIGNATURE OF INVENTOR Juan Jordano Fraga

DATE 26-7-00

09600648-082200